Claims

[c1] 1. A method for real-time detecting and quantifying a nucleic acid template in a PCR mixture comprising the steps of

a) thermally cycling the PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, the nucleic acid template, primers to amplify at least one amplicon from the nucleic acid template, and a double stranded DNA dye, wherein the amplicon has a melting temperature of T_m;

b) obtaining cycle by cycle a pre-T_m emission at a MT below the T_m and a post-T_m emission at the a MT above the T_m;

c) determining cycle by cycle an emission amount of the amplicon, which is the difference between the pre-T_m emission and the post-T_m emission.

[c2] 2. The method of claim 1 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c3] 3. The method of claim 2 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c4] 4. The method of claim 1 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c5] 5. The method of claims 4 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c6] 6. The method of claim 1 wherein the MT below the T_m is 0.25 ^{O}C below, 0.5 ^{O}C below, 1.0 ^{O}C below, 1.5 ^{O}C below, or 2.0 ^{O}C below the T_m.

[c7] 7. The method of claim 1 wherein the MT above the T_m is 0.25 ^{O}C above, 0.5 ^{O}C above, 1.0 ^{O}C above, 1.5 ^{O}C above, or 2.0 ^{O}C above the T_m.

[c8] 8. The method of claim 1 wherein the emission amount of the amplicon is obtained through a computer program which performs a calculation of subtracting the pre-T_m emission from the post-T_m emission or the post-T_m emission from the pre-T_m emission.

[c9] 9. A method for real-time detecting and quantifying a first nucleic acid template and a second nucleic acid template in a PCR mixture comprising the steps of

a) thermally cycling a PCR mixture wherein the PCR mixture comprises a thermostable polymerase, a double stranded DNA dye, the first template and the second template, primers for amplifying a first amplicon from the first template and a second amplicon from the second template, and wherein the first amplicon has a first T_m and the second amplicon has a second T_m and the first T_m is less than the second T_m;

b) obtaining cycle by cycle a first emission at a first MT between an annealing/extension temperature and the first T_m and a second emission at a second MT between the first T_m and the second T_m;

c) determining cycle by cycle a first emission amount of the first amplicon which is the difference between the first emission and the second

emission, and a second emission amount of the second amplicon which is the second emission.

[c10] 10. The method of claim 9 further comprising a step of obtaining cycle by cycle a third emission at a third MT between the second T_m and a total denaturing temperature, wherein the second emission amount is the difference between the second emission and the third emission.

[c11] 11. The method of claim 9 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c12] 12. The method of claim 11 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c13] 13. The method of claim 9 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c14] 14. The method of claims 13 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c15] 15. The method of claim 9 wherein the first MT is 0.25 ^{O}C below the first T_m, 0.5 ^{O}C below the first T_m, 1.0 ^{O}C below the first T_m, 1.5 ^{O}C below the first T_m, or 2.0 ^{O}C below the first T_m, and wherein the first MT is higher than the annealing temperature.

[c16] 16. The method of claim 9 wherein the second MT is 0.25 ^{O}C below the second T_m, 0.5 ^{O}C below the second T_m, 1.0 ^{O}C below the second T_m, 1.5 ^{O}C

below the second T_m, or 2.0 ^{O}C below the second T_m, and wherein the second MT is higher than the first T_m.

[c17] 17. The method of claim 9 wherein the second MT is 0.25 ^{O}C above the first T_m, 0.5 ^{O}C above the first T_m, 1.0 ^{O}C above the first T_m, 1.5 ^{O}C above the first T_m, or 2.0 ^{O}C above the first T_m, and wherein the second MT is less than the second T_m.

[c18] 18. The method of claim 9 wherein the second MT is the first T_m + 0.25^{O}C< the second MT< the second T_m-0.25^{O}C, the first T_m + 0.5^{O}C< the second MT< the second T_m-0.5^{O}C, the first T_m + 1.0^{O}C< the second MT< the second T_m-1.0^{O}C, the first T_m + 1.5^{O}C< the second MT< the second T_m-1.5^{O}C, or the first T_m + 2.0^{O}C< the second MT< the second T_m-2.0^{O}C.

[c19] 19. The method of claim 10 wherein the third MT is 0.25 ^{O}C above the second T_m, 0.5 ^{O}C the second T_m, 1.0 ^{O}C above the second T_m, 1.5 ^{O}C above the second T_m, or 2.0 ^{O}C above the second T_m, and wherein the third MT is less than the total denaturing temperature.

[c20] 20. The method of claim 9 wherein the emission amount of the first amplicon is obtained through a computer program performing a calculation of subtracting the first emission from the second emission or subtracting the second emission from the first emission.

[c21] 21. A method for real-time detecting and quantifying a first nucleic acid template and a second nucleic acid template in a PCR mixture comprising the steps of:

a) thermally cycling a PCR mixture wherein the PCR mixture comprises a thermostable polymerase, a double stranded DNA dye, the first template and the second template, primers for amplifying a first

amplicon from the first template and a second amplicon from the second template, and wherein the first amplicon has a first T_m and the second amplicon has a second T_m and the first T_m is less than the second T_m;

b) obtaining cycle by cycle a first pre-T_m emission at a MT below the first T_m and a first post-T_m emission at the a MT above the first T_m and a second pre-T_m emission at a MT below the second T_m and a second post-T_m emission at the a MT above the second T_m;

c) determining cycle by cycle a first emission amount of the first amplicon which is the difference between the first pre-T_m emission and the first post-T_m emission; and a second emission amount of the second amplicon which is the difference between the second pre-T_m emission and the second post-T_m emission.

[c22] 22. The method of claim 21 wherein the double stranded DNA dye is a double stranded DNA intercalating dye

[c23] 23. The method of claim 22 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c24] 24. The method of claim 21 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c25] 25. The method of claims 24 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c26] 26. The method of claim 21 wherein the MT below the first T_m and/or the second T_m are 0.25 ^{O}C below, 0.5 ^{O}C below, 1.0 ^{O}C below, 1.5 ^{O}C below, or 2.0 ^{O}C below.

[c27] 27. The method of claim 21 wherein the MT above the first T_m and/or the second T_m are 0.25 ^{O}C above, 0.5 ^{O}C above, 1.0 ^{O}C above, 1.5 ^{O}C above, or 2.0 ^{O}C above.

[c28] 28. The method of claim 21 wherein the emission amount of the amplicons is obtained through a computer program performing the calculation of subtracting the pre-T_m emission from the post-T_m emission or subtracting the post-T_m emission from the pre-T_m emission.

[c29] 29. A method for real-time detecting and quantifying a total of *n* nucleic acid templates in a PCR mixture comprising the steps of:

a) thermally cycling a PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, nucleic acid templates including *n* nucleic acid templates, primers for amplifying *n* amplicons, and a double stranded DNA dye;

b) obtaining cycle by cycle a MT_k emission at MT_k and $MT_{(k+1)}$, wherein $T_{m(k-1)} < MT_k < T_{mk} < MT_{(k+1)} < T_{m(k+1)}$, T_{mk} is the T_m of a *k*th amplicon, $T_{m(k-1)}$ is the T_m of a (*k*-1)th amplicon except that $T_{m(k-1)}$ is an annealing and/or an extension temperature when *k*=1, $T_{m(k+1)}$ is the T_m of a (*k*+1)th amplicon except that $T_{m(n+1)}$ is a total denaturing temperature when *k*=n, and *k* and *n* are positive integers, $1 \leq k \leq n$, and $n \geq 2$;

c) determining cycle by cycle an emission amount of the *k*th amplicon which is the difference between the MT_k emission and the $MT_{(k+1)}$ emission.

[c30] 30. The method of claim 29 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c31] 31. The method of claim 30 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c32] 32. The method of claim 29 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.

[c33] 33. The method of claims 32 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c34] 34. The method of claim 29 wherein $T_{m(k-1)} + 0.25^{O}C < MT_k < T_{mk}$, $T_{m(k-1)} + 0.5^{O}C < MT_k < T_{mk}$, $T_{m(k-1)} + 1.0^{O}C < MT_k < T_{mk}$, $T_{m(k-1)} + 1.5^{O}C < MT_k < T_{mk}$, or $T_{m(k-1)} + 2.0^{O}C < MT_k < T_{mk}$.

[c35] 35. The method of claim 29 wherein $T_{mk} + 0.25^{O}C < MT_{(k+1)} < T_{m(k+1)}$, $T_{mk} + 0.5^{O}C < MT_{(k+1)} < T_{m(k+1)}$, $T_{mk} + 1.0^{O}C < MT_{(k+1)} < T_{m(k+1)}$, $T_{mk} + 1.5^{O}C < MT_{(k+1)} < T_{m(k+1)}$, $T_{mk} + 2.0^{O}C < MT_{(k+1)} < T_{m(k+1)}$.

[c36] 36 The method of claim 29 wherein $T_{m(k-1)} < MT_k < T_{mk} - 0.25^{O}C$, $T_{m(k-1)} < MT_k < T_{mk} - 0.5^{O}C$, $T_{m(k-1)} < MT_k < T_{mk} - 1.0^{O}C$, $T_{m(k-1)} < MT_k < T_{mk} - 1.5^{O}C$, or $T_{m(k-1)} < MT_k < T_{mk} - 2.0^{O}C$.

[c37] 37. The method of claim 29 wherein $T_{mk} < MT_{(k+1)} < T_{m(k+1)}-0.25^{O}C$, $T_{mk} < MT_{(k+1)} < T_{m(k+1)}-0.5^{O}C$, $T_{mk} < MT_{(k+1)} < T_{m(k+1)}-1.0^{O}C$, $T_{mk} < MT_{(k+1)} < T_{m(k+1)} -1.5^{O}C$, $T_{mk} < MT_{(k+1)} < T_{m(k+1)}-2.0^{O}C$.

[c38] 38. The method of claim 29 wherein $T_{m(k-1)} + 0.25^{O}C < MT_{k} < T_{mk}-0.25^{O}C$, $T_{m(k-1)} + 0.5^{O}C < MT_{k} < T_{mk} - 0.5^{O}C$, $T_{m(k-1)} + 1.0^{O}C < MT_{k} < T_{mk} - 1.0^{O}C$, $T_{m(k-1)} + 1.5^{O}C < MT_{k} < T_{mk} - 1.5^{O}C$ or $T_{m(k-1)} +2.0^{O}C < MT_{k} < T_{mk} - 2.0^{O}C$.

[c39] 39. The method of claim 29 wherein $T_{mk} + 0.25^{O}C < MT_{(k+1)} < T_{m(k+1)}-0.25^{O}C$, $T_{mk} + 0.5^{O}C < MT_{(k+1)} < T_{m(k+1)}- 0.5^{O}C$, $T_{mk} + 1.0^{O}C < MT_{(k+1)} < T_{m(k+1)}-1.0^{O}C$, $T_{mk} + 1.5^{O}C < MT_{(k+1)} < T_{m(k+1)}- 1.5^{O}C$, or $T_{mk} +2.0^{O}C < MT_{(k+1)} < T_{m(k+1)}-2.0^{O}C$.

[c40] 40. The method of claim 29 wherein $2 \leq n \leq 35$, $2 \leq n \leq 18$, $2 \leq n \leq 10$, $2 \leq n \leq 7$, or $2 \leq n \leq 5$.

[c41] 41. The method of claim 40 wherein n = 2, 3, 4, or 5.

[c42] 42 The method of claim 29 wherein the PCR mixture further comprises a FRET based probe.

[c43] 43. The method of claim 42 wherein the FRET based probe is selected from the group consisting of a Taqman probe, a double-dye oligonucleotide probe, an Eclipse probe, a Molecular Beacon probe, a Scorpion probe, a Hybridization probe, a ResonSense probe, a Light-up probe, and a Hy-Beacon probe.

[c44] 44. The method of claim 29 wherein the PCR mixture further comprises a second primer-based double stranded DNA dye that emits differently from the double stranded DNA dye.

[c45] 45. The method of claim 29 wherein the emission amount of the *k*th amplicon is obtained through a computer program performing the subtraction of MT_k emission from $MT_{(k+1)}$ emission or the subtraction of the $MT_{(k+1)}$ emission from MT_k emission.

[c46] 46. A method for detecting and quantifying a total of *n* nucleic acid templates in multiplex real-time PCR comprising the steps of:

a) thermally cycling a PCR mixture, wherein the PCR mixture comprises a thermostable polymerase, nucleic acid templates including *n* nucleic acid templates, primers for amplifying *n* amplicons, and a double stranded DNA dye;

b) obtaining cycle by cycle a pre-T_{mk} emission of the *k*th amplicon at a MT between $T_{m(k-1)}$ and T_{mk} and a post-T_{mk} emission of the *k*th amplicon at a MT between T_{mk} and $T_{m(k+1)}$, wherein $T_{m(k-1)} < T_{mk} < T_{m(k+1)}$, T_{mk} is the T_m of a *k*th amplicon, $T_{m(k-1)}$ is the T_m of a (*k*-1)th amplicon except that $T_{m(k-1)}$ is an annealing and/or an extension temperature when $k=1$, $T_{m(k+1)}$ is the T_m of a (*k*+1)th amplicon except that $T_{m(n+1)}$ is a total denaturing temperature when $k=n$, and *k* and *n* are positive integers, $1 \leq k \leq n$, and $n \geq 2$;

c) determining cycle by cycle an emission amount of the *k*th amplicon which is the difference between the pre-T_{mk} emission and the post-T_{mk} emission.

[c47] 47. The method of claim 46 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c48] 48. The method of claim 47 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c49] 49. The method of claim 46 wherein the double stranded DNA dye is a primer-based double stranded DNA dye.

[c50] 50. The method of claims 49 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c51] 51. The method of claim 46 wherein the MT between $T_{m(k-1)}$ and T_{mk} is $T_{m(k-1)}$ +0.25^{O}C< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}, $T_{m(k-1)}$ +0.5^{O}C< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}, $T_{m(k-1)}$ +1.0^{O}C< the MT between $T_{m(k-1)}$ and T_{mk}< T_{mk}, $T_{m(k-1)}$ +1.5^{O}C< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}, or $T_{m(k-1)}$ +2.0^{O}C< MT_k < T_{mk}.

[c52] 52. The method of claim 46 wherein the MT between T_{mk} and $T_{m(k+1)}$ is T_{mk} +0.25^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$, T_{mk} +0.5^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$, T_{mk} +1.0^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$, T_{mk} +1.5^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$, T_{mk} +2.0^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$.

[c53] 53. The method of claim 46 wherein the MT between $T_{m(k-1)}$ and T_{mk} is $T_{m(k-1)}$< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}-0.25^{O}C, $T_{m(k-1)}$ < the MT between $T_{m(k-1)}$ and T_{mk}< T_{mk}-0.5^{O}C, $T_{m(k-1)}$< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}-1.0^{O}C, $T_{m(k-1)}$< the MT between $T_{m(k-1)}$ and T_{mk}< T_{mk} –1.5^{O}C, or $T_{m(k-1)}$< the MT between $T_{m(k-1)}$ and T_{mk}< T_{mk}-2.0^{O}C.

[c54] 54. The method of claim 46 wherein the MT between T_{mk} and $T_{m(k+1)}$ is T_{mk}< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$-0.25^{O}C, T_{mk}<the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$-0.5^{O}C, T_{mk}< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$-1.0^{O}C, T_{mk}< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$ -1.5^{O}C, T_{mk}<the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$-2.0^{O}C.

[c55] 55.. The method of claim 46 wherein the MT between $T_{m(k-1)}$ and T_{mk} is $T_{m(k-1)}$ + 0.25^{O}C<the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk}-0.25^{O}C, $T_{m(k-1)}$ + 0.5^{O}C<the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk} - 0.5^{O}C, $T_{m(k-1)}$ + 1.0^{O}C<the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk} - 1.0^{O}C, $T_{m(k-1)}$ + 1.5^{O}C <the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk} - 1.5^{O}C or $T_{m(k-1)}$ +2.0^{O}C< the MT between $T_{m(k-1)}$ and T_{mk} < T_{mk} - 2.0^{O}C.

[c56] 56. The method of claim 46 wherein the MT between T_{mk} and $T_{m(k+1)}$ is T_{mk} + 0.25^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$-0.25^{O}C, T_{mk} + 0.5^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$- 0.5^{O}C, T_{mk} + 1.0^{O}C<the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$- 1.0^{O}C, T_{mk} + 1.5^{O}C < the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$- 1.5^{O}C, or T_{mk} +2.0^{O}C< the MT between T_{mk} and $T_{m(k+1)}$ < $T_{m(k+1)}$- 2.0^{O}C.

[c57] 57. The method of claim 46 wherein $2 \leq n \leq 35$, $2 \leq n \leq 18$, $2 \leq n \leq 10$, $2 \leq n \leq 7$, or $2 \leq n \leq 5$.

[c58] 58 The method of claim 46 wherein the PCR mixture further comprises a FRET based probe.

[c59] 59. The method of claim 46 wherein the FRET based probe is selected from the group consisting of a Taqman probe, a double-dye oligonucleotide probe, an Eclipse probe, a Molecular Beacon probe, a Scorpion probe, a Hybridization probe, a ResonSense probe, a Light-up probe, and a Hy-Beacon probe.

[c60] 60. The method of claim 46 wherein the PCR mixture further comprises a second primer-based double stranded DNA dye that emits differently from the double stranded DNA dye.

[c61] 61. The method of claim 46 wherein the emission amount of the *k*th amplicon is obtained through a computer program performing the subtraction of the pre-T_{mk} emission from the post-T_{mk} emission or the subtraction of the post-T_{mk} emission from the pre-T_{mk} emission

[c62] 62. A computer software program for quantifying a real-time PCR amplicon which, when executed by a computer processor, performs the subtraction of a pre-T_m emission from a post-T_m emission or the subtraction of the post-T_m emission from the pre-T_m emission.

[c63] 63. The computer software program of claim 62 wherein the emission was obtained from a double stranded DNA dye.

[c64] 64. The computer software program of claim 62 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c65] 65. The computer software program of claim 64 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c66] 66. The computer software program of claim 62 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.

[c67] 67. The computer software program of claim 66 wherein the primer-based double stranded DNA dye is selected from the group consisting of

fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c68] 68. The computer software program of claim 62 wherein a pre-T_m emission is obtained at a MT below the T_m of the amplicon and a post-T_m emission is obtained at a MT above the T_m.

[c69] 69. The computer software program of claim 68 wherein the MT below the T_m is 0.25 ^{O}C below, 0.5 ^{O}C below, 1.0 ^{O}C below, 1.5 ^{O}C below, or 2.0 ^{O}C below the T_m.

[c70] 70. The computer software program of claim 68 wherein the MT above the T_m is 0.25 ^{O}C above, 0.5 ^{O}C above, 1.0 ^{O}C above, 1.5 ^{O}C above, or 2.0 ^{O}C above the T_m.

[c71] 71. The computer software program of claim 62 which is stored and/or executed in a PCR instrument.

[c72] 72. The computer software program of claim 62 which is stored and/or executed in a computer connected to a PCR instrument.

[c73] 73. A computer program product comprising a computer memory having a computer software program, wherein the computer software program, when executed by a computer processor, performs the subtraction of a pre-T_m emission from a post-T_m emission or the subtraction of the post-T_m emission from the pre-T_m emission.

[c74] 74. The computer program product of claim 73 wherein the emission was obtained from a double stranded DNA dye.

[c75] 75. The computer program product of claim 73 wherein the double stranded DNA dye is a double stranded DNA intercalating dye.

[c76] 76. The computer program product of claim 75 wherein the double stranded DNA intercalating dye is selected from the group consisting of ethidium bromide, YO-PRO-1, Hoechst 33258, SYBR Gold, and SYBR Green I.

[c77] 77. The computer program product of claim 73 wherein the double stranded DNA dye is a primer-based double stranded DNA dye that is covalently linked to the primers.

[c78] 78. The computer program product of claim 77 wherein the primer-based double stranded DNA dye is selected from the group consisting of fluorescein, FAM, JOE, HEX, TET, Alexa Fluor 594, ROX, and TAMRA, rhodamine, BODIPY-FI.

[c79] 79. The computer program product of claim 73 wherein a pre-T_m emission is obtained at a MT below the T_m of the amplicon and a post-T_m emission is obtained at a MT above the T_m.

[c80] 80. The computer program product of claim 79 wherein the MT below the T_m is 0.25 ^{O}C below, 0.5 ^{O}C below, 1.0 ^{O}C below, 1.5 ^{O}C below, or 2.0 ^{O}C below the T_m.

[c81] 81. The computer program product of claim 79 wherein the MT above the T_m is 0.25 ^{O}C above, 0.5 ^{O}C above, 1.0 ^{O}C above, 1.5 ^{O}C above, or 2.0 ^{O}C above the T_m.

[c82] 82. The computer program product of claim 73 which is stored and/or executed in a PCR instrument.

[c83] 83. The computer program product of claim 73 which is stored and/or executed in a computer connected to a PCR instrument.

[c84] 84. A PCR instrument comprising the computer program product of claim 73.